IMMUNOLOGICAL RESPONSES IN SKIN TOLERANT, CELLULAR CHIMERIC CHICKENS

by

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TABLE OF CONTENTS

INTRODUCTION

Billingham *et al.* (1956) defined acquired immunological tolerance as a central failure of the mechanism of immunological response. This originally included the concept of a specific inhibition of response which depends on the embryo's inability to respond and its subsequent inability to do so. Tolerance produced in an embryo or newborn animal and in an adult animal appears to be comparable in effect but differs in the amount of antigen required for induction (Brent and Gowland 1962, 1963). Complete tolerance would encompass the inability to form humoral antibodies and inhibition of other cellular immunological activities against donor antigens, i.e., those which cause rejection of dissociated cells, skin and other solid grafts and are responsible for graft-versus-host reactions when such cells are injected into immunologically defenseless recipients. The dissociation of humoral reactions and cellular reactions has been postulated (Stark *et al.*, 1960, 1961a, 1961b, 1962).

Subbarayudu (1967) observed that chickens made tolerant to skin grafts by injections of whole blood during the embryonic and newly hatched period were capable of reacting against the same donor antigens. Blood of skin tolerant birds injected into an embryo of the same genotype as the original donor caused a graft-versus-host reaction measured in terms of splenomegaly. The objective of this study was to investigate the state of tolerance of skin tolerant birds in respect to humoral and cellular reactivity and chimeral status.

REVIEW OF LITERATURE

Induction and Maintenance of Tolerance

Brent and Gowland (1963) listed the requirements for induction of tolerance in an embryonic or newborn animal. Tolerance is due to the introduction of a foreign antigen and the resulting unresponsiveness is specific for the antigen to which tolerance is induced. Persistence of the antigen is necessary for maintenance of tolerance. When the tolerance-inducing stimulus is in the form of viable cells, the host becomes a cellular chimera (Gowland, 1965). Brent and Gowland (1962, 1963) in experiments with adult animals showed that the difference between induction of tolerance in neonatal and in immunologically competent mice was quantitative. To overcome mature antibody-forming cells, cellular antigen must be administered over a prolonged period if donor and host differ at the H-2 locus, whereas a single injection of cells into a new-born mouse will give prolonged tolerance of skin grafts across the same H-2 barrier. Stark et al., (1962) reported the best method of inducing tolerance in newly hatched chicks is by simultaneous injection of blood and transplantation of a skin graft from a newborn donor. Hasek (1956) found embryonic parabiosis to be effective in birds. Subbarayudu (1967) found that the age of the donor had no significant effect on the induction of tolerance in chicks. Bainbridge et al., (1966) concluded that the intravenous route was more suitable for the induction of tolerance because a high proportion of the antigen reaches the lymphoid tissue.

Argyris (1965b) stated that homograft tolerance produced by neonatal injection of lymphoid cells is more successful in some strains of mice than others. Resistance has been attributed to the advanced stage of immunological maturity of neonatal hosts and the genetic relationship between host and donor. Responsiveness was measured by injecting spleen cells from newborn mice into F_1 animals and testing the ability of the injected cells to elicit a graft-versus-host reaction in terms of spleen and liver enlargement.

Billingham et al., (1956) found that the power to confer tolerance to skin resides entirely in the leucocyte fraction of blood. Plasma and red blood cells were ineffective. Schierman and Nordskog (1964) reported that no tolerance for skin was produced in chicks with repeated injections of red blood cells. The animals showed a humoral unresponsiveness and failed to clear donor cells from the blood. Tolerance of skin grafts can be induced by spleen cells, leucocytes, lymph node cells and bone marrow cells (Stark et al., 1962). Terasaki (1959b) found that in the chicken, monocytes and thymocytes could be used and were as effective as blood lymphocytes.

Billingham and Silvers (1961b) reported lymph node cells as being most effective in inducing tolerance in mice although splenic cells and leucocytes had similar abilities. Contrarily, Argyris (1964a) reported that spleen cells were more effective than node cells in inducing tolerance in mice. Terasaki (1959b) observed that tolerance induction was independent of a graft-versus-host reaction when he used F_1 adult cells, which are immunologically incapable of reacting against

a parental host, to produce tolerance in chickens.

Argyris (1964a) found that cell dosage affected the incidence but not the duration of tolerance. The incidence of tolerance depends on the age of the host at injection, the type and dose of inoculated cells and the route of injection. Variation in the length of the tolerant state could be due to variation in development of the immune activity of the host and subsequent initiation of a host-versus-graft reaction. Argyris (1965a) reported that homograft tolerance in C3H mice injected neonatally with CBA spleen cells was spontaneously lost with age. The immunological reactivity of host lymphoid cells directed against the graft antigens increased as loss of tolerance proceeded. Billingham et al., (1953) stated that mice and chickens never develop or develop only to a limited degree the power to react immunologically against foreign homologous tissue cells of which they were inoculated in fetal life.

Zaalberg and van der Meul (1966) injected parental bone marrow cells into lethally irradiated F_1 mice. These cells apparently became tolerant of F_1 antigens. This was shown by transferring bone marrow and lymph node cells from the F_1 to an irradiated parental strain mouse and testing for tolerance with a skin graft. Tolerance was not shown if bone marrow cells only were transferred. Loss of tolerance by cells removed from the presence of the antigen in the F_1 host when transferred to the original donor strain was explained by the replacement of the tolerant cells by nontolerant cells. Loss of tolerance did not occur if the thymus was removed.

Using C3H and A strain mice and their F_1 hybrid, Martinez et al. (1959), found that if tolerance is induced by either parental or F_1 hybrid cells, the recipient will accept grafts from the parental strain or F_1 hybrids. They concluded that the F_1 shares all histocompatibility antigens present in each parental strain. No new antigens appear in the hybrid from genic interaction. However Billingham and Silvers (1961b) found node cells from hybrid mice were less effective than cells of homologous origin.

Levels of Tolerance

Three ways to show acquired immunological tolerance are acceptance of an appropriate skin graft, failure to produce antibodies against the specific antigen and failure to produce a graft-versus-host reaction on the chorioallantoic membrane (CAM) (Hilgard et al., 1962). Splenomegaly tests and related assays of the graft-versus-host reaction, failure to clear erythrocytes from the blood, and the persistence of injected cells have also been used as tests of the state of tolerance. A completely tolerant animal should be unable to react in any of these test situations.

Goodman (1965) regarded parental strain mice given F_1 hybrid spleen cells at birth which retained skin grafts greater than 15 days but less than 100 days as partially tolerant. Fully tolerant mice retained grafts more than 100 days. Hemagglutination and cytotoxic tests were used to demonstrate the presence of cellular grafts. Donor cells were found in the peritoneal cavities and in lymph nodes but not in

marrow. There was an almost complete lack of red cell chimerism and an inability of tolerant mice to produce circulating isoantibodies. Stark et al., (1962) considered partial tolerance in chickens as graft survival for about 8 weeks.

Prehn and Main (1954) first demonstrated a gene dosage effect in skin graft rejections. They found, using BALB/c and BALB/c x DBA/2 F_1's, that heterozygous F_1 grafts survive longer than homozygous grafts on DBA/2 mice. There was no difference when F_1 and DBA/2 grafts were placed on BALB/c mice. Schierman and Nordskog (1964) showed that F_1 leucocytes induced a higher degree of tolerance to F_1 grafts than to grafts from the homozygous B-locus incompatible partner strain. Tolerance to homozygous skin grafts increased as the dose of F_1 cells increased. They found equal acceptance of F_1 and parental grafts on F_1 hosts. G B-1 chickens became fully tolerant to G B-2 grafts with multiple injections of F_1 leucocytes, thereby relating level of tolerance to the dose of B antigens in the graft.

Lapp and Bliss (1967) studied the effects of allelic dosage and graft size on survival in untreated mice differing at the H-1 locus. Survival of Cc grafts on cc hosts was significantly longer than CC grafts. Increasing the size of the heterozygous graft increased survival time. They postulated that there was a difference in vulnerability to attack of homozygous and heterozygous grafts but that homozygous and heterozygous cells had the same competence in inducing tolerance or immunization. Galton (1967) studied the rejection of skin incompatible at the H-3 locus. He observed longer survival of

F_1 grafts than homozygous grafts in C57BL/10J and B10.LB mice.

Split Tolerance

Stark and Frenzl (1959) and Stark *et al*. (1960, 1961a, 1961b) induced tolerance to skin grafts in chicks at hatching. Stark *et al*. (1962) grouped them according to manifestation of tolerance after challenge with donor blood as follows:

1. complete tolerance- complete inhibition of isohemagglutinin formation and prolonged tolerance of skin

2. split tolerance- isohemagglutinin formation and prolonged tolerance of skin

3. a temporary crisis in the graft followed by recovery and tolerance of the graft

4. loss of tolerance- complete destruction of the graft and agglutinin formation.

On the basis of these results it was suggested that the two types of immunological reaction, i.e., antibody formation and a delayed type of cellular hypersensitivity were dissociated. It was thus shown that humoral antibody formation is less easily inhibited than is the cellular reaction. Hasek *et al*., (1966) reported that after exchanging skin grafts among C line chickens, agglutinins were found in 15% of all birds. Active immunization with erythrocytes from the graft donor gave 100% with hemagglutinins in animals that rejected grafts and 16% with hemagglutinins in animals that accepted grafts. These results also suggest a dissociation of response and less inhibition of the humoral response.

Billingham *et al*. (1965) defined split tolerance as a differential unresponsiveness on the part of the host to some of the foreign transplantation antigens represented in neonatally inoculated homologous cells. Split tolerance as defined in this manner was first observed by Billingham and Brent (1959) in A strain mice made tolerant of CBA but not of C57 antigens by injection of CBA x C57 cells. Chimeral tests showed after both hybrid and C57 skin grafts were rejected that the hosts still possessed spleen cells of donor origin. They hypothesized that persisting cells responsible for chimerism are located in situations that protect them from specific sensitivity on the part of the host. Brent and Courtenay (1962) also observed that spleens of animals with split tolerance had cells of CBA and C57 specificity. In two cases where both skin grafts were rejected, the animals were found to be chimeric for C57 and CBA antigens. It has been found easier to induce tolerance in newborn A mice with CBA rather than C57 cells. It was suggested that suppression of the surface C57 antigens of some hybrid cells in such an environment had occurred. Greater immunological susceptibility of hybrid skin than of hybrid spleen cells has also been suggested.

Weissman (1966) observed that tolerance is usually directed towards the antigen to which tolerance can be achieved with greater ease when tolerance is directed to a portion of the total antigenic inoculum. Tolerance to the male-specific antigen was induced with BALB/c x C57BL male spleen cells or C3H male spleen cells injected into BALB/c x C57BL females. Tolerance to C3H skin grafts was not

observed. Tests for persistence of male-specific antigen consisted of testing for transfer of tolerance in newborns or persistence of male-specific sensitizing antigen in adults. Male-specific antigen persisted in split tolerant mice in very low concentration. Abolishing tolerance by transfer of cells immunized to the male-specific antigen was thought to abolish the lymphoid chimerism of the host. Martinez and Smith (1964) reported that split tolerance to the Y-linked antigen can be induced in mice by injecting the animals at birth with subcellular antigenic material from male spleen cells. It was suggested that injected viable cells may be destroyed by the host but release enough transplantation antigen to overcome the host's resistance to Y-linked antigen.

Billingham and Silvers (1962b) introduced the term "restricted tolerance" to the phenomenon whereby homologous cells may induce high degrees of tolerance to themselves but not to other tissues of identical genetic constitution. BN rats which were feebly tolerant or intolerant of Lewis skin following neonatal injection with F_1 leucocytes, spleen cells or node cells still had donor cells in their blood and solid tissue after they had rejected their test grafts. Failure of a cellular inoculum to confer tolerance of skin does not exclude the possibility that it has conferred tolerance to some cells present in the neonatal inoculum. Where chimerism persists after a skin graft has been rejected, only a low degree of tolerance was initially induced. Presumably, the host could reject a skin graft but not long-established foreign cells disseminated throughout its tissues.

Graft-Versus-Host Reactions

A graft-versus-host reaction (GVH) ordinarily implies a tolerant or immunologically incompetent host which cannot eliminate injected cells. One of the manifestations of complete tolerance is the inability of a tolerant animal to cause a GVH reaction in an embryo having the antigen to which tolerance was induced.

Simonsen (1957) found that adult spleen cells could give a GVH reaction when injected into chick embryos or newborn mice. The leucocyte portion of blood was also found to cause spleen enlargement in chickens. Using inbred mice, he noted that splenomegaly occurred only if the donor was genetically different from the recipient. It was assumed that the reaction consisted of the formation of serum antibodies and of cellular immunity. Simonsen believed pyroninophilic cells derived from the donor were involved and concluded that splenomegaly was a direct result of donor cell proliferation.

Cock and Simonsen (1958) and Isacson (1959) reported no splenomegaly in chickens if host and donor belonged to the same inbred strain. Jaffe and McDermid (1962) reported that no splenomegaly occurs if donor and host have the same B blood group in chickens. No enlargement occurs unless the host contains B antigens foreign to the donor. Biggs and Payne (1960) reported that blood was equivalent to spleen cells in evoking a GVH reaction. Simonsen (1957) and Isacson (1959) found that spleen cells and peripheral blood cells acquired immunological competence for this reaction around the tenth day after hatching, and Solomon (1961)

found 5-day-old spleen cells effective. Solomon (1963, 1964), using the spleen assay technique in 14-day chick embryos, showed that peripheral blood contained immunologically competent cells two days after hatching and that even 13 to 15 day-old embryos may be capable of an active but weak immune response. Isacson (1959) and Solomon and Tucker (1961) found that embryos injected at 13-15 days gave more splenic enlargement in 6 days than if injected at 11 or 17-20 days. Solomon (1962) reported greater spleen weights for female hosts in some strains when injected with spleen cells or whole blood.

Simonsen (1957) showed that the cell responsible for the GVH reaction could be serially transferred through nine passages and thus eliminated the polymorphonuclear leucocyte which does not reproduce and suggested the lymphocyte or monocyte. Terasaki (1959a) using preparations of these cells concluded that the lymphocyte was responsible. Gowans (1965) cited two papers to show the role of small lymphocytes in GVH reactions. Gowans (1962) showed that when parental small lymphocytes were injected into F_1 rats, they homed into the lymphoid tissue of the host, and a proportion changed into pyroninophilic cells which divided. Small lymphocytes from tolerant animals which had long-standing homografts of skin, failed to cause any signs of GVH when injected intravenously into the skin donors (Gowans et al., 1963). He believes that the component of the homograft reaction and the GVH reaction is the large pyroninophilic cell.

Biggs and Payne (1959) used the sex chromosome difference to identify male cells in spleens of female chicks embryos inoculated

with blood. Male cells were found in all 15 female spleens examined. In nine female embryos, 75 male and 95 female cells were found in metaphase. This suggested that splenic enlargement was provoked by host as well as donor cells. Mun et al. (1959) found that irradiation which blocked or inhibited mitosis removed the growth-stimulating ability of grafted tissue. He concluded that donor cells capable of mitosis are necessary for an enlarged host spleen. Howard et al., (1961) observed that irradiation of the host prior to grafting inhibited cellular multiplication in the GVH reaction. Seto and Albright (1965) analyzing donor and host contributions to splenic enlargement in chick embryos found an equivalent reduction in splenic enlargement resulting from X-irradiation of either donor spleen cells or the recipient embryos.

Owen et al. (1965) used the sex chromosome as a marker to study the GVH reaction in the chick embryo. Donor cells form a progressively greater proportion of cells in the spleen as incubation proceeds. Three days after injection, 8% of the dividing cells were of donor origin. By six days after injection, 93% were of donor origin and donor proliferation exceeded that of the host. When histocompatibility differences exist between donor and host, spleens of embryos of similar incubation time contain similar proportions of donor cells despite considerable variations in overall spleen weight. The amounts of RNA, DNA and protein per gram wet weight of spleen remains constant throughout splenic enlargement suggesting that increased spleen size is due directly to cellular proliferation. The constancy in cell population suggests both donor and host proliferation are interdependent. The overall rate of

cell proliferation is related to the degree of histocompatibility of donor and host.

Simonsen (1962) stated that in general there is a correlation between the number of cells injected and the severity of changes. The strength of antigenic stimulus and age are also involved. Seto and Albright (1965) stated that for embryos of any age, the degree of splenic enlargement is proportional to the number of donor cells inoculated. Simonsen (1962) found that within a dose range of 1/2 to 5 million cells, there is a good linear relationship between log dose and spleen index. Jaffe and Fechheimer (1966) found that the increase in donor cells corresponds to the logarithmic growth phase of spleen enlargement.

In certain GVH reactions, a gene dosage effect may be demonstrated. Simonsen and Jensen (1959) as described by Simonsen (1962) found a higher spleen index when AKR cells were injected into C3H mice than when injected into C3H x AKR newborns.

Hilgard _et al_., (1962) reported a method of showing acquired immunological tolerance by a graft-versus-host reaction on the CAM of chick embryos. A cell suspension of embryonic tissue (CC) was injected into embryos (AA) of approximately the same age. These chicks were hatched and their blood was tested for the ability to cause a reaction on the CAM of CC and CA embryos. Of 17 embryos injected, only 3 showed no evidence of tolerance. Where tolerance was established, it diminished with age. Michie _et al_., (1961) showed that spleen cells from adult A mice made tolerant of CBA antigens failed to give spleen enlargement in

newborn CBA mice.

Argyris (1964b) used the capacity of C3H spleen and lymph node cells to elicit splenomegaly in allogeneic newborn CBA mice as an index of immune status. GVH reaction was elicited by normal node cells or presensitized spleen cells. Node cells from mice fully tolerant of skin grafts were less active than normal node cells. During the spontaneous loss of tolerance, spleen and node cells acquired some reactivity. The subnormal immunologic status of post tolerant C3H mice was confirmed by delayed rejection of second and third CBA skin grafts and by the ease with which tolerance could be reinduced. Hitological analysis of lymphoid tissues demonstrated the development of an immunologic reaction in lymph nodes of 4 month-old tolerant mice before external indications of graft rejection. The peak of GVH reactions in mice is reached when donors are 3 to 6 month old. Significant differences in reaction were noted between 1/2-/ and 1-month-old donors and 2-/ and 3-/ or 6-month-old donors. The capacity of tolerant lymphoid cells to react as measured by GVH is subnormal but increases during and after graft rejection.

Van Bekkum _et al_., (1965) studied parental to F_1 irradiation chimeras and concluded that parental CBA donor cells which repopulate the lymphatic tissues of CBA x C57BL hosts become specifically tolerant to host type antigens as demonstrated by GVH assays in newborn CBA x C57BL mice. Transfer of cells to irradiated CBA mice transferred tolerance to C57BL skin grafts. Where microscopic examination of the grafts showed a weak homograft reaction was occurring, the GVH reaction

was positive but not maximal. When skin grafts showed a high degree of tolerance, the spleen cells had regained at least partially the capacity to react. He concluded that the GVH reaction was at least as sensitive as microscopical examination of skin grafts.

Chimeral Status

Billingham _et al_., (1956) using lysis to test for red cell chimerism found that chimerism persisted only where the tolerance of skin homografts was still complete. They noted that disappearance of graft tolerance and chimerism does not mark a return to normal reactivity. Ford _et al_., (1956) found a strain of mice with a chromosome marker that could be used to identify donor cells and observed dividing cells of donor type in the spleen, lymph nodes and thymus of injected mice. Hort _et al_., (1961) studying erythrocyte chimerism in grafted chickens by agglutination methods and clearance of $Na_2{}^{51}CrO_2$ labeled erythrocytes observed erythrocyte chimeras where grafts persisted for an extended period and also where the graft was slowly destroyed. It was noted that several months after disappearance of erythrocyte chimerism, antibody formation was still inhibited. Michie _et al_., (1961) reported that an animal which maintains a donor type skin graft may be a lymphoid cellular chimera harboring cells from the original donor.

Trentin and Session (1962) tested mice made tolerant to skin by neonatal injections of spleen cells with a T6 chromosome marker for chimeral status. Donor cells were found in the bone marrow,

lymph nodes, spleen and thymus in animals fully tolerant of donor skin or undergoing breakdown of tolerance. No donor cells were found where grafts had been rejected. This suggested to them that disappearance of donor lymphocytes precipitated loss of donor skin grafts. Host cells appeared to remain tolerant only as long as donor lymphoid cells persisted.

Mitchison (1962) reported that erythrocyte elimination could detect departures from full tolerance which are too slight to cause rejection of skin grafts. He noted that partial tolerance can last indefinitely and is not a stage during the breakdown of tolerance. Billingham and Silvers (1962a) injected Lewis rat cells into BN rats and found that some BN animals made feebly tolerant of Lewis skin by F_1 leucocytes at birth were chimeric in regard to blood leucocytes after rejection of Lewis grafts. Some BN hosts which gave no evidence of tolerance were found to be leucocyte chimeras several weeks after test operations with skin. Strain A mice injected with C57 x CBA spleen or marrow cells were not completely tolerant of C57 or F_1 skin but were highly tolerant of CBA skin. Such animals were found to be cellular chimeras possessing foreign cells of donor origin presumably having C57 and CBA antigens after rejection of C57 or F1 skin. Spleen cells from these tolerant animals induced tolerance to CBA skin when put into newborn A mice. Silverman and Chin (1962) found circulating cells of rat bone marrow in 50% of mice that rejected rat skin transplants. Mice reverting to a nontolerant state had a high titer of hemagglutinins to rat red blood cells.

Martinez and Good (1963) were able to transfer tolerance to isologous mice with spleen cells from mice injected with allogenic spleen cells neonatally and postulated the persistence and replication of cells in the lymphoreticular system of the host. Brent and Gowland (1963) found that CBA cells injected into A mice may persist when unresponsiveness is incomplete and may persist after destruction of the original hybrid skin graft but not after transplantation of a second skin graft.

Argyris (1964a) by radioautography found donor cells in the spleen up to the sixth day after injection when it was presumed that labeling was lost due to isotope dilution after repeated cell division. Chimera analysis, by testing for sensitization by cells from tolerant animals, showed donor lymphoid cells were present in most C3H mice before, during and shortly after graft rejection. Chimerism was lost in most post-tolerant mice which had rejected a second CBA graft. McKhann (1964) reported lymphoid chimerism of mice made tolerant of H-2 or H-3 differences. Wilson and Talmage (1965) found that 53 of 54 mice injected with spleen and bone marrow cells neonatally and skin grafted after maturation were erythrocyte chimeras after retaining grafts for a minimum of 100 days.

Billingham _et al_., (1965) reported a sensitive test for chimerism that could detect the presence of as few as 12,500 Y-antigen containing cells in a standard population of 20 million spleen cells. A cell suspension from an adult injected into a normal adult should sensitize if chimeral cells are present. Cells injected into neonatal hosts should

induce tolerance. The possibility of an antigen chimera rather than a cellular chimera is lessened with the latter system because of the difficulty in inducing tolerance with non-living material. As few as 3×10^5 isologous male leucocytes were sufficient to induce tolerance in 100% of neonatal C57 females. By this system, 0.5% was the minimum level of lymphoid cell chimerism that could be detected. All females made tolerant of male skin by neonatal injection of 5 million isologous spleen cells were chimeras. Abolition of tolerance could be affected by transfer of isologous female sensitized lymphoid cells if a demonstrable level of chimerism was present so adoptive immunization was also used as a test for cellular chimeras. If transfer of one to three immune equivalents of cells to a tolerant C57 female gave destruction of a tolerated graft, it was inferred as being a chimera. Failure to abolish tolerance was considered evidence of a low level or no chimerism.

Silobrcic (1965) using a sensitization test found that all mice injected neonatally with spleen cells whether highly tolerant or non-tolerant to skin grafts were cellular chimeras. Persistence of donor cells was observed 35 days after rejection of a second graft.

Skin Grafting

Prendergast (1964) showed that cells from the regional lymph node invade a homograft of skin in substantial numbers using tritiated thymidine to label the lymph nodes. Gowans (1965) implicated lymphocytes in the reaction against homografts by procedures such as thymectomy that prevent development of lymphoid tissue or remove small lymphocytes.

Such procedures impair the ability to reject homografts. Billingham *et. al.*, (1956) showed that injections of normal lymph node cells could abolish tolerance to skin grafts in mice suggesting that the deficiency of the tolerant animal lies in its lymphoid tissue. Peripheral blood leucocytes also restore a tolerant animal. Micklem and Brown (1961) concluded that the breakdown of first set skin homografts depended on the participation of sensitized cells. Mice were seen to reject a first set graft without forming circulating hemagglutinins. Silverstein and Kramer (1965) in studies with fetal lambs which can reject skin grafts after midgestation showed that homograft rejection can be accomplished without the formation of plasma cells in the graft or in reactive lymph nodes draining the graft site. Lambert and Frank (1967) concluded from the observation of more proliferation of endothelial cells and fibroblasts at the site of allografts than autografts that allogeneic differences can be recognized and responded to locally almost at once. Gleason and Fanguy (1964) obtained results that suggested the presence of circulating donor type red cell agglutinins in the host do not influence skin homograft survival. Shabart *et. al.*, (1966) concluded from the time differences between the duration of transplantation immunity and the appearance of hemagglutinins that hemagglutinins do not play a major role in graft rejection.

The sensitivity of skin graft survival as a test for tolerance has been questioned and the possibility of adaptation of grafts has been studied by a number of workers. Billingham *et. al.*, (1956) stated that the homograft reaction is the most accurately reproducible of any

of immunology with a standard deviation of $\pm$ 10%. They regarded skin as the test of greatest sensitivity and maintained that tolerance is systemic and that a tolerated graft does not build up a privileged position in the host. The fact that a tolerant host can be caused to reject a long-tolerated homograft by passive immunization shows that the antigenicity of the tolerated graft remains unchanged. They also found rejection of a later homograft by a host apparently tolerant of an earlier homograft occurs only when tolerance is incomplete.

Hasek (1961) suggested adaptation of grafts from the finding that a second graft transplanted from the same donor within a short time after the first has the same fate as the first. If the second graft is transplanted more than six months after the first, its fate may be different. Stark et al., (1962) found that repeated grafting from the original donor before and after injection of donor blood was in most cases successful. In four cases, both grafts were destroyed as tolerance was abolished. Serum immunity against blood cells did not prevent a primary take or prolonged survival of grafts. Transplantation of skin grafts surviving in tolerant mice for 330 days were made back to the original donor strain with permanent survival (Haskova et al., 1965). Grafts from such tolerant animals transplanted to normal animals of the original recipient strain were rejected as fresh grafts. Second grafts put on tolerant mice persisted.

Billingham et al., (1965) found that transplanting a second male skin isograft to tolerant, presumed non-chimeric C57 females gave indefinite survival of second grafts giving further proof of tolerance.

Transfer of immune lymphoid cells led to shorter survival of secondary grafts than primary grafts. Haskova and Hinzova (1966) grafted skin from neonatal mice followed by a second graft from adult mice 20 to 30 days later and found a second set reaction. When the time interval between grafting was over 60 days, a first set rejection or prolongation was observed. Zaalberg and van der Meul (1966) observed that some mice rejected a second graft when the first graft remained normal and concluded a healthy graft is not a guarantee of complete tolerance of the host. A healed graft is thought to be more resistant to a weak immune reaction than newly grafted skin.

MATERIALS AND METHODS

Two inbred lines, RPL-6 and R were used along with their F_1 hybrid. RPL-6 is a White Leghorn obtained from the U.S.D.A. Poultry Research Laboratory, East Lansing, Michigan. It has been under the effects of close inbreeding since 1939 (Crittenden et al., 1964). The B histocompatibility locus is segregating, but B^{13}/B^{13} embryos were used exclusively to test for relative reactivity of leucocytes. Line R is a Brown Leghorn imported from the Poultry Research Center, Edinburgh, Scotland and has an inbreeding coefficient greater than 0.76. It is thought to be homozygous at the B histocompatibility locus from the results of a large number of skin graft exchanges within the line (Bacon 1967).

The skin tolerant birds tested in the experiments described were R line birds which had been made tolerant to B^{13}/B^{13} and B^{R}/B^{13} skin grafts by the intravenous injection of 0.20 ml of whole blood from F_1 hybrid B^{R}/B^{13} birds as 14-day-old embryos by Subbarayudu (1967). Injected birds were tested for tolerance by skin grafting at 16-17 days of age. Grafts were from chicks of the same age and were approximately 2cm x 1 1/2cm in size. Experiments to test for completeness of tolerance were begun after grafts had been maintained in good condition for 7 months. Controls were R line birds which had received no treatment. Examination of the original grafts up to a year after grafting and after the experiments reported here were completed showed good survival of both grafts and feathering. One bird sacrificed for spleen cell injections after 11 months was showing a slight reaction against the B^{R}/B^{13} graft.

Natural matings provided fertile eggs for experiments. Eggs were accumulated over a week's period and then set in incubators. All chicks were pedigree hatched and wingbanded for identification.

White blood cells were used for most embryonic injections. Blood was drawn from the brachial vein or by cardiac puncture into a heparinized syringe and transferred to a centrifuge tube. The blood was centrifuged and most of the plasma was removed. The white blood cells formed a layer on top of the packed red cells and were resuspended in the small amount of plasma left by swirling the plasma with a Pasteur pipette. The cells in the plasma were removed with the pipette and suspended in saline solution. The red cells were washed 2 to 3 times with saline and the white cell layer removed each time. Excess red blood cells were removed from the white cell suspension by allowing erythrocytes to settle out of suspension by standing or by using slight centrifugation. The white cell suspension was spun down and resuspended in Hank's solution. A cell count was made in a Levy-Hausser counting chamber and appropriate dilutions were made with Hank's solution. Cells were injected into 13-day-old embryos for splenomegaly tests or into 15-day-old embryos for tolerance induction.

Embryos were injected as reported by Subbarayudu (1967) which was an adaptation of the procedure of Billingham (1961). The eggs were candled and the position of a chorioallantoic vein was marked. The shell over the vein was removed by cutting a small rectangle in the shell with a fine toothed hack saw blade and lifting the shell with a sharp pointed lancet. Sterile mineral oil was placed on the membrane to make the vein visible.

Using a micromanipulator, a 30 gauge needle on a 1/4 ml syringe was used to inject directly into the vein. After injection, tape was placed over the window in the shell. Sterilized equipment was used and injections were performed under a chemistry hood which was sterilized by ultraviolet light preceding use.

An assay of the GVH reaction was made using weights of spleens from embryos injected at 13 and sacrificed at 19 days of incubation. Spleens were removed from the embryos and weighed immediately on a Mettler balance to the nearest one tenth of a milligram. Sex of the embryo was determined by examination of the gonads.

Spleen cell suspensions for tolerance induction were prepared by peeling the membrane from the spleen and teasing it with forceps in saline until much of the connective tissue could be removed. The cells were centrifuged and then gently resuspended by drawing the solution into a syringe with progressively smaller needles from size 20 until they would pass easily through a 30 gauge needle. The cells were then concentrated by centrifugation and resuspended in a known volume of Hank's solution and counted.

A grafting technique described by Bacon (1967) using adult wattle as donor tissue was used to test for transfer of tolerance. Hosts were grafted at 10 or 17 days of age. Flexible collodion was applied to the backs of the chicks to stiffen the skin and down. Chicks were previously anesthesized with 0.03 ml of sodium pentobarbitol (commercial "Halatol" containing one grain sodium pentobarbitol per cc which was diluted 1:5 with physiological saline) solution per 10

grams of body weight. Four square sites approximately 1 x 1 cm were made on each recipient. Wattle tissue was cut from appropriate donors and split by pulling and slitting with a scalpel blade. The tissue was placed on filter paper soaked with physiological saline and cut to appropriate graft size. Each recipient received four grafts each of different genotype. Graft sites were assigned at random. Curad bandages 3/4 x 1" were used to protect the grafts. Bandages were removed 5 days after the operation and grafts were scored daily for at least 15 days after which readings were made once or twice a week. Grafts were scored on a system similar to that of Polley <u>et al</u>., (1960) as presented in Table 1.

Serum from skin tolerant birds was obtained by drawing 15 ml of blood into a heparinized syringe and transferring to a centrifuge tube. The blood was centrifuged to separate the plasma from the cells. The plasma was removed from the top with a Pasteur pipette and defibrinated by shaking with small glass beads for 10 to 15 minutes.

To test for possible differences between the GVH reactivity of leucocytes of line R birds made tolerant of B^{13}/B^{13} and B^{R}/B^{13} skin grafts by embryo injections of B^{R}/B^{13} blood and untreated line R birds, B^{13}/B^{13} embryos of line RPL-6 were injected with white blood cells at 13 days of incubation. Four embryos were injected with white blood cells for each dosage level of 50,000; 100,000; 200,000; 400,000; 800,000 from each of 10 skin tolerant R birds and 10 untreated R birds used as positive controls. The spleens of injected embryos were weighed 6 days later. Hank's solution injected and uninjected embryos were also opened and

Table 1. Macroscopic numerical scoring system used to estimate the severity of the homograft reaction.

Score	Description
6	Smooth, bright, healthy appearing.
5	Smooth, but some discoloration and/or inflammation apparent.
4	Moderate discoloration and may be slightly shrunken.
3	Discolored and shrunken.
2	Discolored, much shrunken, crusty, and becoming detached at edges.
1	Graft sloughed.
X	Graft missing but not sloughed (faulty operative technique or accidental loss).

spleens weighed on the same day as negative controls.

To test for the presence of chimeral cells in skin tolerant birds, concentrated white blood cell suspensions were made from the same birds used in the GVH assay and injected into 15-day-old R embryos. The chicks were hatched and grafted at 10 or 17 days to test for induction of tolerance. Chicks injected with white cells from untreated birds were grafted at the same time along with uninjected chicks. One control chick grafted at 10 days retained a B^R/B^{13} graft, presumably made tolerant by the presence of the skin graft, so 17-day-old chicks were used for subsequent grafting. Billingham et al., (1956) and Cannon and Longmire (1952) showed that 2-week-old chicks were immunologically mature. Billingham stated that 2-week-old chicks are far beyond the

stage of development at which transplantation of a homograft can of itself confer tolerance. Chicks received skin grafts of the following genotypes: R B^R/B^R, R x RPL-6 B^R/B^{13}, RPL-6 B^{13}/B^{13}, and RPL-6 B^{12}/B^{13}. They will subsequently be referred to as B^R/B^R, B^R/B^{13}, B^{13}/B^{13}, and B^{12}/B^{13}, respectively.

Relatively small numbers of R chicks were available for test grafting due to the high rate of embryonic death following injection of large numbers of cells. Seto and Albright (1965) also found a high frequency of embryonic death within a short time following inoculation of large numbers of donor cells. When more than 10^6 cells per gram embryo weight were injected, death occurred with a high frequency. Death was immediate and accompanied by severe systemic hemorrhage.

To estimate the number of donor-type cells present in skin tolerant birds, R embryos were injected with varying doses of B^R/B^{13} white blood cells at 15 days of incubation and test grafted at 17 days of age. Spleen cells were also used in one experiment to test for the presence of chimeral cells and to see whether there was a difference in the ability of the two cell types in tolerance induction.

Stark et al., (1962) reported that chicks with split tolerance had sera which had a neutralizing effect on lymphoid cells from the skin donor. He found that sera with a titer over 1:32 prevented a GVH reaction after incubation with cells injected into newly hatched chicks. One million B^{13}/B^{13} white blood cells were therefore incubated for an hour with sera from line R skin tolerant birds designated as $B^R/B^R(B^R/B^{13})$ to see if antibodies against the B_{13} antigen could be

detected. The cells were then concentrated by centrifugation, resuspended and 100,000 cells injected into each of six B^{12}/B^{12} embryos for each serum donor on the 13th day of incubation. Embryos were opened on the 19th day and the spleens weighed. The same number of $B^{13}B^{13}$ cells was also incubated with sera from untreated R birds and with known anti-13 sera for a positive control.

RESULTS

Leucocyte Reactivity of Skin Tolerant Birds

Splenomegaly tests were conducted to look for differences in reactivity of leucocytes of skin tolerant and untreated birds. One skin tolerant bird and one untreated bird were tested at the same time. Tests continued over a period of weeks until 10 pairs were compared. Four B^{13}/B^{13} embryos were injected at 13 days of incubation and spleen weights compared on the 19th day of incubation for each of the five dosage levels from each donor.

Results are shown in Table 2. Within the dosage levels used the skin tolerant birds consistently produced splenomegaly but at lower levels than untreated birds. A quadratic equation was calculated from these values for each bird and the number of cells necessary to elicit a 50% of maximum spleen enlargement, approximated at 65 milligrams, was calculated by plotting log dose against spleen weight. Table 3 shows the number of cells estimated from the equations as necessary to elicit a 65 mg. spleen. Equations for the pooled results of both groups were also derived and the number of cells required is shown. Ratios of the number of cells required for skin tolerant and untreated birds are also shown in Table 3. Approximately five times as many cells were required from skin tolerant birds as from untreated birds to elicit a 65 mg. spleen. A paired comparisons t-test on the differences between the number of cells required for a 65 mg. spleen for skin tolerant and untreated birds revealed a significant difference ($P < .01$).

Table 2. Mean spleen weights obtained by injecting leucocytes from $B^R/B^R(B^R/B^{13})$ skin tolerant (T) and untreated B^R/B^R birds into B^{13}/B^{13} embryos at 5 dosage levels.[1]

Wing Band	Sex	Cell Dosage 50,000	100,000	200,000	400,000	800,000
T 4783	♂	13.6[2]	20.7	69.2	130.4	107.5
3731		49.0	84.5	127.2	109.0	136.4
T 6726	♂	21.4	33.0	28.4	83.2	112.4
3649		31.6	49.5	66.6	95.5	149.8
T 6723	♂	20.1	22.2	61.5	97.4	80.1
3650		57.2	128.0	139.2	132.5	123.0
T 6722	♀	35.1	31.1	42.4	120.3	137.4
3978		68.7	136.6	108.2	205.5	143.6
T 6743	♀	35.1	59.0	43.0	80.2	132.7
3632		56.2	50.0	106.8	126.0	103.7
T 5105	♂	17.5	25.1	15.0	70.3	109.0
3803		100.7	143.9	251.2	133.4	193.9
T 6990	♀	24.6	28.9	62.7	88.7	93.8
3973		51.0	90.3	112.0	94.1	106.1
T 4883	♀	14.2	17.3	66.1	58.1	84.7
3772		55.0	43.2	82.1	21.2	123.4
T 6725	♂	17.2	16.4	19.0	26.4	52.5
3727		32.9	28.7	60.1	55.1	81.6
T 6727	♀	23.9	22.5	41.5	74.2	31.8
3799		26.4	47.3	48.4	81.8	113.7
T grand average		22.1	28.2	45.3	83.9	101.1
grand average		55.8	83.9	117.0	108.0	129.0

[1]Mean spleen weights of 12.1 and 11.8 mg. were obtained from 31 and 33 embryos injected with Hank's solution and uninjected, respectively.

[2]Means are based on spleen weights at 19 days of incubation obtained from survivors of 4 embryos injected at 13 days of incubation. Most embryos survived over this period.

Table 3. Comparison of the number of cells required from skin tolerant and untreated birds to elicit a 65 mg. spleen.

	Estimated Number of Cells[1]		
Pair	Skin tolerant birds	Untreated birds	Ratio
1	159,000	65,000	2.45
2	347,000	193,000	1.80
3	222,000	51,000	4.35
4	236,000	47,000	5.02
5	270,000	76,000	3.55
6	426,000	36,000	11.83
7	235,000	62,000	3.79
8	288,000	44,000	6.54
9	1,134,000	444,000	2.57
10	--	265,000	--
Pooled	312,000	66,000	4.70

[1]Estimates were derived from quadratic equations calculated using the mean spleen weights at the 5 dosage levels.

Test for the Presence of Humoral Antibody

Sera from skin tolerant $B^R/B^R(B^R/B^{13})$ birds were incubated with B^{13}/B^{13} cells before their injection into B^{12}/B^{12} embryos with results as shown in Table 4. Known immune sera specific for B^{13} cells neutralized these cells so that no spleen enlargement occurred when they were injected into B^{12}/B^{12} embryos. There was no such effect with sera from each of 2 skin tolerant or of 2 untreated birds. Antibody against the

Table 4. Results of neutralization tests for humoral antibody.

Serum from	+	Cells	--->	Embryos[1]	Mean Spleen Weights	
none	+	none	--->	B^{12}/B^{12}		10.7
immune anti-13	+	B^{13}/B^{13}	--->	B^{12}/B^{12}		10.9
$B^R/B^R(B^R/B^{13})$	+	B^{13}/B^{13}	--->	B^{12}/B^{12}	69.3	57.3
$B^R/B^R(B^R/B^{13})$	+	B^{13}/B^{13}	--->	B^{12}/B^{12}	45.3	
B^R/B^R	+	B^{13}/B^{13}	--->	B^{12}/B^{12}	36.2	48.2
B^R/B^R	+	B^{13}/B^{13}	--->	B^{12}/B^{12}	60.3	

[1]Six embryos were injected for each serum donor and for the known immune serum. Three uninjected embryos were opened for controls.

B_{13} antigen was not detected.

Test for the Presence of Chimeral Cells

Concentrated white blood cells from skin tolerant birds were injected into B^R/B^R embryos at 15 days of incubation. If chimeral cells of B^R/B^{13} genotype were present, they would be expected to induce tolerance to the B_{13} antigen in the embryo. After hatching, the injected chicks were tested for tolerance by B^R/B^{13} and B^{13}/B^{13} skin grafts.

There appeared to be transfer of tolerance to 3 of 4 chicks grafted at 10 days of age that were injected with 10×10^6 white blood cells from skin tolerant birds at 15 days of incubation. One of 3 control birds injected with 10×10^6 cells from an untreated line R bird

accepted a B^R/B^{13} graft suggesting that tolerance might have been induced with skin grafts in a few cases. Therefore the results on birds grafted at 10 days were considered as possibly confounded and subsequently birds were grafted at 17 days only.

Table 5 summarizes the results obtained from skin grafts placed on 17-day-old chicks. All control chicks had rejected their B^R/B^{13} and B^{13}/B^{13} skin grafts before the 14th postoperative day. Continued graft survival beyond the 13th day was therefore assumed due to the induction of tolerance. Of chicks receiving 20 to 160 million cells from B^R/B^R (B^R/B^{13}) donors, 5 of 8 retained the B^{13}/B^{13} grafts and 6 of 8 retained the B^R/B^{13} grafts. These results are interpreted to indicate that chimeral B^R/B^{13} cells were present in the inoculum from line R skin tolerant birds.

Incompatible B^R/B^{13}, B^{13}/B^{13}, and B^{12}/B^{13} grafts on untreated R chicks and chicks injected as embryos with cells from untreated line R birds, were rejected between the 7th and 14th day after grafting. There were 57 such grafts on 19 birds. All birds received a B^R/B^R intraline graft, and all accepted it through the 14th postoperative day. The prompt rejection of B^{12}/B^{13} grafts on chicks accepting B^R/B^{13} and B^{13}/B^{13} skin indicated that tolerance was specific for the B_{13} antigen for which tolerance was induced. One of 41 birds injected with cells from skin tolerant or F_1 donors failed to reject the B^{12}/B^{13} graft. He retained all four grafts in good condition at day 14.

Table 5. Number of skin grafts surviving (score ≥ 3) / total grafts on the 14th postoperative day on line R birds.

Injected Cells	B^{13}/B^{13}	%	B^{R}/B^{13}	%
NONE	0/15	0	0/15	0
B^{R}/B^{R}	0/4	0	0/4	0
$B^{R}/B^{R}(B^{R}/B^{13})$	5/8[1]	62	6/8	75
F_1 white cells				
10,000	0/5	0	1/5	20
50,000	2/5	40	5/5	100
100,000	1/5	20	4/5[1]	80
200,000	2/5	40	5/6	83
1-3,000,000	0/4	0	4/4	100
F_1 spleen cells				
10,000	0/4	0	3/4[1]	75
1-3,000,000	0/5	0	0/4	0

[1] One additional graft was rejected by 21 days.

Quantitative Estimate of Chimeral Status

Results of skin grafting line R birds injected as embryos with varying doses of F_1 cells should allow estimation of the number of chimeral cells present in the tolerance inducing inoculum from skin tolerant birds. Table 5 shows the percentage of B^{R}/B^{13} and B^{13}/B^{13}

grafts surviving on the 14th postoperative day. From these results, it was estimated that there were more than 200,000 chimeral cells present in the inoculum from $B^R/B^R(B^R/B^{13})$ birds. Forty per cent survival of B^{13}/B^{13} grafts and 83% survival of B^R/B^{13} grafts was obtained with 200,000 F_1 cells. Cells from chimeric birds induced tolerance for 62.5% B^{13}/B^{13} grafts and 75% B^R/B^{13} grafts. Estimates based on numbers of cells injected indicated that at least 0.1% to 0.5% chimeral cells were present in the circulating leucocytes of skin tolerant birds tested.

Graft survival after injection of spleen cells was not as good as when the same number of white blood cells from the circulating blood were injected. Only one experiment was conducted with spleen cells, and it is possible that excessive handling in dissociating the cells may have resulted in a less viable suspension.

DISCUSSION

Line R B^R/B^R birds injected with 0.2 ml of B^R/B^{13} whole blood at 14 days of incubation were still tolerant of B^{13}/B^{13} and B^R/B^{13} skin grafts a year later. Chimeral tests showed that B^R/B^{13} cells or antigens derived from such cells were still present in the circulating blood of skin tolerant birds in numbers sufficient to induce tolerance in 15-day-old R embryos. The evidence suggests therefore that these chickens were tolerant of dissociated B^R/B^{13} cells in the peripheral circulation. It would be expected from tolerance studies by Hilgard et al., (1962) with chicks, Michie et al., (1961) with mice and Gowans et al., (1963) with rats that leucocytes from these birds if completely tolerant of the B_{13} antigen would not react against B^{13}/B^{13} cells of embryos. Contrary to expectation, splenomegaly tests showed that such cells consistently produced GVH reaction but to a lesser degree than cells from untreated R birds. Tests for serum antibody against the B_{13} antigen were negative.

It has been suggested that injected cells of the tolerance-inducing inoculum are less sensitive to rejection than skin grafts (Brent and Courtenay 1962, Billingham et al., 1965). These cells may be located in situations that protect them from reaction by the host. The presence of chimeral cells in the circulating blood of our chickens argues against a protective or inaccessible site. It has also been suggested that a long-tolerated skin graft may persist where a slight reaction which can destroy a second graft is taking place

(Zaalberg and van der Meul 1966). There is convincing evidence that the humoral response is less easily inhibited than the cellular response (Stark <u>et</u> <u>al</u>., 1962). Van Bekkum <u>et</u> <u>al</u>., (1965) working with mice considered the GVH reaction to be at least as sensitive for detecting breakdown of tolerance as microscopical examination of skin grafts. CBA cells injected into irradiated (CBA x C57BL) F_1's become tolerant of host antigens as demonstrated by the lack of a GVH reaction when spleen cells were injected into newborn F_1's. Cells transferred from the F_1 to irradiated CBA mice transferred tolerance to C57BL skin grafts. Animals bearing such skin grafts in good condition or undergoing a weak reaction nevertheless possessed spleen cells capable of producing GVH reactions when injected into CBA x C57BL newborns. Our results provide confirmatory evidence that the GVH reaction is also a more sensitive criterion for detecting lack of complete tolerance in the chicken than the other methods mentioned.

Tests of tolerance <u>within</u> skin tolerant birds, i.e., persistence of chimeral status and absence of humoral antibodies, indicated that they were completely tolerant. The GVH reaction is a test in which immunologically competent cells are removed from the animals and put into a different environment which may be more favorable or stimulte proliferation. Tolerance of these cells within the skin tolerant bird may be dependent in some way on the environment. Even when removed from the skin tolerant chicken, a maximum response was not obtained from such leucocytes. This quantitative difference suggests that either a permanent change in number of competent cells or an inhibition of the

immunological capacity of individual cells had occurred. A large amount of antigen may be necessary to stimulate such cells into division or reaction.

The B_{13} antigen present within the skin tolerant birds may not be recognized as foreign. The fact that birds tolerating skin grafts can produce antibodies as shown by Stark _et al._, (1962) and Hasek _et al._, (1966) is consistent with the view that the humoral response is less easily inhibited. If humoral antibody were present at levels too low to be detected by the technique used, an interaction with donor cells may be responsible for failure of recognition by cells of the host.

As long as sufficient antigenic cells are present to maintain tolerance in replacement cells, tolerance to skin grafts or chimeral cells should remain. If the antigen were to be diluted so that new cells were not made tolerant, gradual loss of tolerance would be expected. If only a portion of the total cell population is tolerant the results may be more readily seen in the sensitive GVH reaction. Loss of skin grafts and of chimeral cellular grafts would depend on the relative vulnerability of the two types of grafts to attack by nontolerant cells.

Data on line R chickens made tolerant by F_1 cells to the B_{13} antigen showed that tolerance to F_1 skin grafts was induced more often than to the homozygous B^{13}/B^{13} skin grafts. A dosage effect is postulated as described by Schierman and Nordskog (1964). They suggested that since twice as many foreign antigen sites were present on homozygous cells than F_1 cells, a quantitative difference in immune response may be present.

SUMMARY

Line R birds (B^R/B^R) injected with F_1 hybrid B^R/B^{13} whole blood as embryos were tolerant of the B_{13} antigen by the criterion of persistence of B^R/B^{13} and B^{13}/B^{13} skin grafts. Transfer of tolerance to line R embryos by leucocytes and spleen cells from skin tolerant birds showed the persistence of B^R/B^{13} chimeral cells in the circulating blood of skin tolerant chickens. Minimal estimates of 0.1% to 0.5% chimeral cells were obtained.

Splenomegaly tests showed however that tolerance was not complete. A quantitative assay of the GVH reaction against B^{13}/B^{13} embryos by leucocytes of skin tolerant and of untreated line R birds showed a consistent but lower response by skin tolerant birds.

A neutralization test was used to look for antibody in skin tolerant birds specific for the B_{13} antigen but no antibody was detected.

All tests of tolerance within the skin tolerant birds indicated a state of complete tolerance. Leucocytes removed from the birds and injected into B^{13}/B^{13} embryos showed incomplete tolerance. It appears from these results that the GVH reaction is a more sensitive criterion for detecting lack of complete tolerance than the other methods mentioned. It appears that there are nontolerant cells within the skin tolerant birds which can be detected when cells are placed in a more favorable environment.

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IMMUNOLOGICAL RESPONSES IN SKIN TOLERANT, CELLULAR CHIMERIC CHICKENS

by

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Line R birds made tolerant of B^{13}/B^{13} and B^{R}/B^{13} skin grafts as 14-day-old embryos by injection of 0.20 ml whole blood from F_1 hybrid B^{R}/B^{13} donors were studied for completeness of tolerance. It had been noted previously that blood from these presumed tolerant birds caused splenomegaly when injected into B^{13}/B^{13} embryos.

To look for possible differences in reactivity between these skin tolerant birds and untreated line R birds, a quantitative graft-versus-host (GVH) assay was applied. Thirteen-day-old embryos were injected with 50,000; 100,000; 200,000; 400,000; and 800,000 white blood cells from skin tolerant and untreated line R birds. The spleens were removed on the 19th day of incubation and weighed. Cells from skin tolerant birds consistently produced splenomegaly but at lower levels than untreated birds suggesting a lower reaction by leucocytes of skin tolerant birds.

The presence of humoral antibody against the B_{13} antigen was sought by means of a neutralization test. Sera from the skin tolerant birds were incubated with B^{13}/B^{13} cells for an hour and then injected into B^{12}/B^{12} embryos. If antibody specific for the B_{13} antigen was present, the B^{13}/B^{13} cells should not react to cause splenomegaly. No evidence of antibody against B_{13} was observed.

To look for the presence of chimeral cells in skin tolerant birds, concentrated white blood cells from these birds were injected into 15-day-old line R embryos. The chicks were hatched and tested for transfer of tolerance by wattle-on-back grafts at 17 days of age. Survival of B^{R}/B^{13} and B^{13}/B^{13} grafts suggested that chimeral cells

were present in skin tolerant birds. On the basis of tolerance induction with varying doses of F_1 cells, it was estimated that there were at least 0.1% to 0.5% chimeral cells present in the circulating blood of skin tolerant chickens.

Spleen cells were used in one experiment to transfer tolerance and for F_1 injections. Inferior results may have been caused by excessive handling in dissociating cells.

Results from induction of tolerance with F_1 white blood cells suggest longer survival of F_1 grafts than homozygous B^{13} grafts on line R birds. A dosage effect seems probable.